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Association of transcobalamin c. 776C>G with overall survival in patients with primary central nervous system lymphoma

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Abstract: Background: Chemotherapy for primary central nervous system lymphoma (PCNSL) is based on methotrexate (MTX), which interferes with both nucleic acid synthesis and methionine metabolism. We have reported previously that genetic variants with influence on methionine metabolism are associated with MTX side effects, that is, the occurrence of white matter lesions as a sign of MTX neurotoxicity. Here, we investigated whether such variants are associated with MTX efficacy in terms of overall survival in MTX-treated PCNSL patients. **Methods:** We analysed seven genetic variants influencing methionine metabolism in 68 PCNSL patients treated with systemic and facultative intraventricular MTX-based polychemotherapy (Bonn protocol). **Results:** Median age at diagnosis was 59 years (range: 28-77), 32 patients were female. Younger age (Wald=8.9; P=0.003) and the wild-type C (CC) allele of the genotype transcobalamin c (Tc2). 776C>G (Wald=6.7; P=0.010) were associated with longer overall survival in a multivariate COX regression analysis. **Conclusion:** This observation suggests that the missense variant Tc2. 776C>G influences both neurotoxicity and efficacy of MTX in the Bonn PCNSL protocol.

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**Association of transcobalamin c. 776C>G with overall survival in patients with primary
central nervous system lymphoma**

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Running title: Transcobalamin c.776C>G and survival in pCNSL

Keywords: primary central nervous system lymphoma; polymorphism; survival

Abstract

Introduction: Chemotherapy for primary central nervous system lymphoma (PCNSL) is based on methotrexate (MTX), which interferes with both nucleic acid synthesis and methionine metabolism. We have reported previously that genetic variants with influence on methionine metabolism are associated with MTX side effects, i.e. the occurrence of white matter lesions as a sign of MTX neurotoxicity. Here we investigated whether such variants are associated with MTX efficacy in terms of overall survival in MTX-treated PCNSL patients.

Methods: We analyzed seven genetic variants influencing methionine metabolism in 68 PCNSL patients treated with systemic and facultative intraventricular MTX-based polychemotherapy (Bonn protocol).

Results: Median age at diagnosis was 59 years (range: 28-77), 32 patients were female. Younger age (Wald = 8.9; $p = 0.003$) and the wild-type C (CC) allele of the genotype transcobalamin c. 776C>G (Wald = 6.7; $p = 0.010$) were associated with longer overall survival in a multivariate COX regression analysis.

Conclusion: This observation suggests that the missense variant transcobalamin c. 776C>G influences both neurotoxicity and efficacy of MTX in the Bonn PCNSL protocol.

Introduction

Primary central nervous system lymphomas (PCNSL) represent approximately 2.4% of all primary brain tumors in the United States (Central Brain Tumor Registry of the United States, 2004-2007). Untreated PCNSL has a poor prognosis of a median survival of only a few weeks. Treatment protocols include chemotherapies and brain irradiation. All first-line chemotherapy protocols in PCNSL are based on high-dose methotrexate (MTX), a competitive inhibitor of dihydrofolate reductase (DHFR), which synthesizes 5,10-methylenetetrahydrofolate (5,10-MTHF; **Figure 1** (Finkelstein, 1990). Thereby, MTX inhibits nucleic acid synthesis. However, 5,10-MTHF is also used for the synthesis of 5-methyltetrahydrofolate (5-MTHF) by 5,10-MTHF-reductase (MTHFR). Together with vitamin B12, which is transported by transcobalamin 2 (Tc2), 5-MTHF serves as cofactor of methionine synthase, which synthesizes methionine from homocysteine. Methionine can be activated to S-adenosylmethionine, which acts a methyl group donor, e.g. for CNS myelination. The reduced folate carrier 1 (RFC1) is involved in the uptake of folate and MTX into the CNS.

Variants of genes encoding these enzymes, transporter and carrier proteins of methionine metabolism are associated with MTX neurotoxicity as indicated by CNS white matter hyperintensities in T2 weighted magnetic resonance imaging (MRI) in PCNSL patients (Linnebank *et al*, 2007; Linnebank *et al*, 2009; Linnebank *et al*, 2005). In the present study we aimed at investigating whether these variants are also associated with the overall survival of PCNSL patients.

Methods

We analyzed 68 consecutive PCNSL patients treated with the Bonn protocol with (n = 42) or without (n = 26) intraventricular therapy (Pels *et al*, 2009; Pels *et al*, 2003). Median age at diagnosis was 59 years (range: 28-77), 32 patients were female. Median Karnofsky score was 70 (range: 50-100). Median time of follow-up was 44 months (range: 1-114). The MTX-based Bonn protocol with and without intraventricular treatment has been described in detail (Pels *et al*, 2009; Pels *et al*, 2003). In brief, the protocol consisted of six chemotherapy cycles with intravenous high-dose MTX (cycles 1,2,4,5), high-dose cytarabine (ara-C; cycles 3,6) and vinca-alkaloids, oral dexamethasone and (until 2002) intraventricular MTX, ara-C and prednisolone. Whereas 62 patients received the complete protocol, 6 patients terminated early because of progressive disease. In the entire population of patients intended to be treated, we analyzed seven genetic variants influencing methionine metabolism by PCR amplification of genomic DNA prepared from peripheral leucocytes and subsequent endonuclease restriction, where applicable. Amplicons were analyzed by agarose gel electrophoresis as published: CBS 844ins68, DHFR c.594+59del19bp, MTHFR c.677C>T, MTHFR c.1298A>C, MTR c.2756A>G, RFC1 c.80G>A, Tc2 c.776C>G (Linnebank *et al*, 2007; Linnebank *et al*, 2009; Linnebank *et al*, 2005).

Multivariate COX regression analysis (forward stepwise; Wald) was used to analyze the association of age, gender, Karnofsky score before treatment, type of Bonn protocol (with or without intraventricular therapy) and the seven variants of methionine metabolism with overall survival as the primary parameter of interest. In addition, the association of the genetic variants with further dependent variables was tested in exploratory analyses, together with the same co-variables as before: treatment response (complete remission, CR, including complete remission unconfirmed, Cru, versus incomplete response/ others; multinominal regression analysis), response duration (COX regression), time to treatment failure (COX regression), and Karnofsky performance score (ANOVA) (Pels *et al*, 2009; Pels *et al*, 2003). Overall survival was

determined from time of histological diagnosis to date of death. Response duration was calculated from the time when response was first documented to relapse. Time to treatment failure was calculated from initiation of treatment to date of disease progression, relapse, death or premature termination of therapy due to any cause (Pels *et al*, 2003). Response was categorized according to a consensus of the International PCNSL cooperative group (Abrey *et al*, 2005). Median overall survival was not reached at the time of this analysis; thus, we provide mean values where applicable. Deviations of genotype distributions from the Hardy Weinberg equation were tested by a χ^2 goodness-of-fit test. Univariate Log Rank analyses with Kaplan Meier curves were used to illustrate data. ANOVA was used to exclude significant differences of age between groups defined by Tc2 genotypes. Threshold was defined with $\alpha < 0.05$. All patients or their legal trustees gave informed written consent to this study, which was approved by the local ethics committee.

Results

Genotyping succeeded for all patients. No genotype distribution deviated from the Hardy-Weinberg equation. In multivariate COX analysis of the intent-to-treat-population, younger age was significantly associated with longer survival (Wald = 6.4; $p = 0.011$), but not clearly with higher Karnofsky performance score before therapy (Wald = 2.7; $p = 0.098$). Gender showed no significant association in this population (Wald = 0.21; $p = 0.649$). In addition, the wild-type C allele of Tc2 c.776C>G was associated with overall survival suggesting a gene-dose effect (Table 1; Figure 2). Of the group of patients with the CC genotype, two patients died due to tumour progression. Of the group with the CC genotype with CG genotype, four patients died to tumour progression, one patient due to therapy-associated toxicity, and three patients probably due tumour progression, which was not proved by imaging. Of the group with the GG genotype, five patients died due to tumour progression. Of those six patients who did not complete treatment

due to early progression, each two carried the genotypes CC, CG and GG not suggesting that the Tc2 genotype was associated with incomplete treatment.

In explorative secondary analyses, patients with the wild-type CC genotype seemed had a longer response duration (median not yet reached; mean: 74 ± 11 months) and a longer time to treatment failure (61 ± 12 months) than the other patients (43 ± 9 and 38 ± 7 , respectively; not significant).

Further, 14 of the 20 patients (0.70) homozygous for the wild-type CC genotype of Tc2

c.776C>G had a complete remission compared with only 24 of the 48 patients (0.50) with the mutant CG or GG genotypes, but this was not significant, either. In addition, the wild-type C allele was associated with a better Karnofsky performance score after treatment for trend ($p =$

0.054; Table 2). None of the patients who completed therapy suffered from overt

neurotoxicity. However, several patients developed white matter changes visible in T2 FLAIR MRI as published previously. If development of such white matter changes was set as

covariable in the COX analysis, the association of Tc2 c.776C>G with overall survival was still significant ($p=0.03$), whereas the other genotypes still did not show significant associations (not shown). The patient groups defined by Tc2 genotypes did not differ in age, CC (56 ± 11 years), CG (58 ± 12), GG (56 ± 14 ; $p=0.722$).

Discussion

Current MTX-based treatment protocols for PCNSL achieve high response rates and clearly have improved prognosis. However, a substantial fraction of patients experience serious adverse events during treatment, have incomplete responses only or suffer from relapses. Individual factors like age and Karnofsky performance score prior to treatment are predictive concerning the clinical outcome (Abrey *et al*, 2006; Ferreri *et al*, 2003; Jahnke & Thiel, 2009). This was confirmed in our study (Karnofsky performance score: trend only) arguing for the representativity of the study population. Another predictive factor, i.e. early complete response to

initial treatment (Pels *et al*, 2010) was not analyzed in this study. The identification of additional factors like genetic variants may help to further improve individual prediction of outcome for different therapeutic options.

In the present study, we report that the mutant G allele of Tc2 c.776C>G was associated with shorter overall survival of PCNSL patients treated with the high-dose MTX-based Bonn protocol (Table 1). We have reported previously that this mutant G allele is associated with MTX-neurotoxicity as indicated by white matter changes detected in T2-weighted magnet resonance imaging MRI (Linnebank *et al*, 2009; Linnebank *et al*, 2005). Interestingly, in the present study, we also observed an association of this mutant variant with lower Karnofsky performance scores after therapy for trend as a further possible indicator of the relevance of this variant for MTX toxicity. Alternatively, the lower Karnofsky performance score in carriers of the G-allele may also be explained by reduced therapeutic efficacy of MTX as suggested by the data summarized in Table 2.

Tc2 is the transporter protein of cobalamin (vitamin B12), which is necessary to remethylate methionine and S-adenosylmethionine from homocysteine. S-adenosylmethionine is needed to synthesize e.g. myelin basic protein, phospholipids and sphingomyelins. Such molecules are necessary for CNS integrity and myelination, and, accordingly, deficiency of S-adenosylmethionine can cause demyelination of the central nervous system (Figure 1) (Surtees *et al*, 1991). The missense mutant G variant of Tc2 c.776C>G (p.P259R) lowers the affinity of Tc2 to cobalamin and leads to reduced concentrations of blood Tc2-cobalamin complexes, e.g. reducing the biological availability of cobalamin for methionine and S-adenosylmethionine synthesis (von Castel-Dunwoody *et al*, 2005). This may explain why the mutant G-allele of Tc2 c.776C>G is associated with progressive demyelination in patients with X-chromosomal

adrenoleukodystrophy and the occurrence of white matter T2 hyperintensities on brain MRI in PCNSL patients treated with the MTX-based Bonn protocol (Linnebank *et al*, 2009; Linnebank *et al*, 2005; Semmler *et al*, 2009). During MTX treatment, patients with one or two mutant G alleles and, as consequence, with a lower availability of cobalamin for 5-MTHF-dependent methionine synthesis will experience pronounced MTX-induced S-adenosylmethionine depletion leading to a higher risk of neurotoxicity and a lower consumption of 5,10-MTHF for 5-MTHF synthesis (Figure 1). In parallel, more 5,10-MTHF might be left for nucleic acid synthesis, possibly decreasing MTX efficacy as one possible explanation of an association with overall survival observed in this cohort (Figure 2c). In our study, additional explorative analyses, which were not powered to yield significant results with respect to the small subgroup sizes, suggested that the association of Tc2 c.776C>G with overall survival may be explained by both, a modification of instantaneous MTX treatment efficacy and toxicity. This is indicated by the post-treatment Karnofsky performance score and by sustained treatment efficacy, as indicated by the time to relapse and time to treatment failure. As limitations, the results of our study remain to be retested in an independent population of PCNSL patients and, preferably, also other MTX-treated populations. We cannot exclude that other factors like differences in the MTX concentrations in the target tissue have confounded our findings. Additional mechanisms including an interaction between Tc2 c.776C>G and rescue with leucovorin (formyltetrahydrofolate), which is converted to 5,10-MTHF antagonizing MTX, may also be relevant, but were not analyzed in our study. Further, the biological consequences of Tc2 c.776C>G may be modified by vitamin B12 or folate plasma levels (Stanislawska-Sachadyn *et al*, 2010), which were not measured in our study population.

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The authors report no conflicts of interest.

Figures

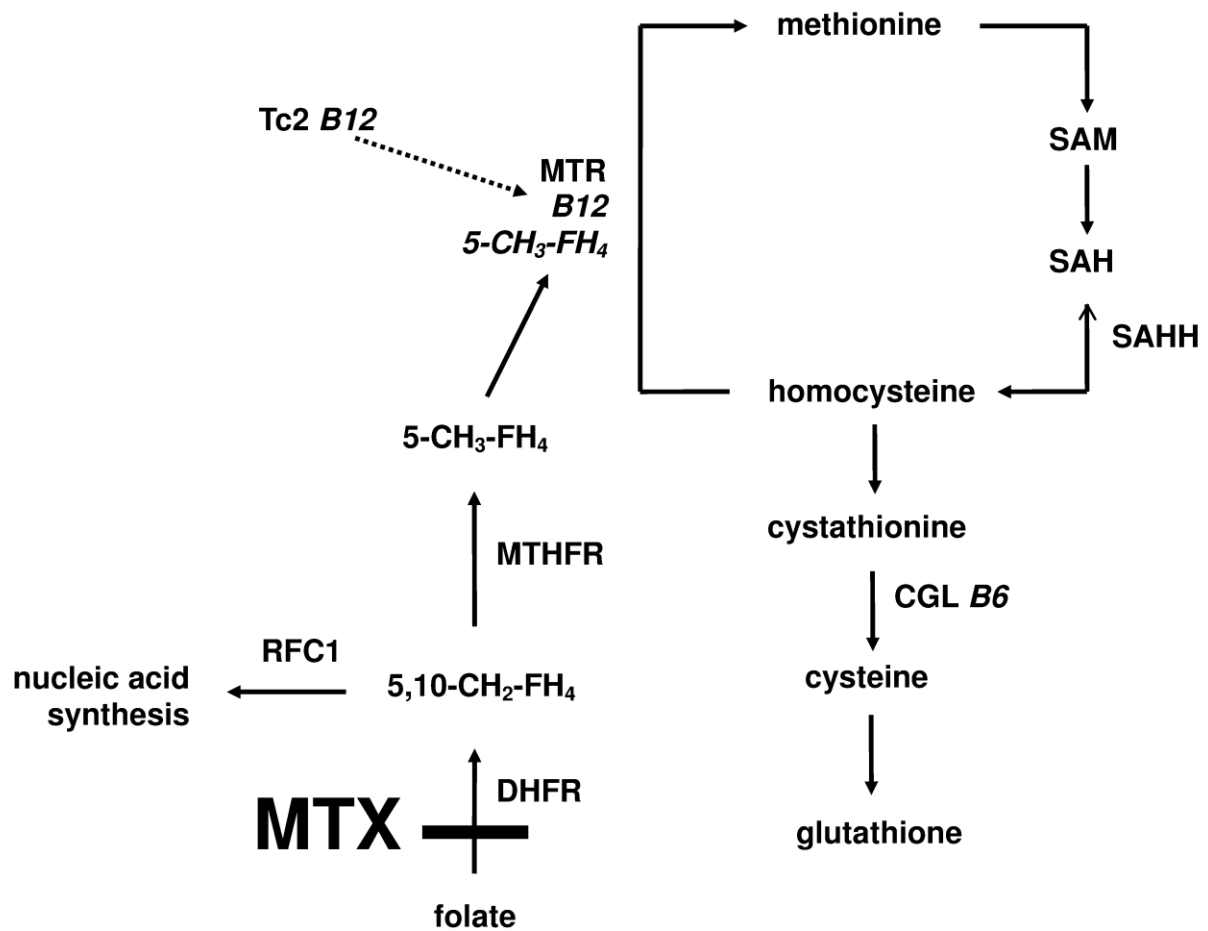


Figure 1 – Human methionine metabolism

Methotrexate (MTX) inhibits dihydrofolate reductase (DHFR), which synthesizes 5,10-CH₂-FH₄ from the folate pool in two subsequent steps. The reduced folate carrier 1 (RFC1) is involved in the uptake of 5,10-CH₂-FH₄ and MTX to tissues and organs including the central nervous system. 5,10-CH₂-FH₄ is needed for nucleic acid synthesis and, alternatively, can be reduced by methylenetetrahydrofolate reductase (MTHFR) to 5-methyltetrahydrofolate (5-CH₃-FH₄). Together with methylcobalamin, 5-CH₃-FH₄ is used by 5-methyltetrahydrofolate-homocysteine S-methyltransferase (MTR, also called methionine synthase) to synthesize methionine.

Transcobalamin 2 (Tc2) is the major transporter protein for cobalamin. Activated methionine (S-adenosylmethionine, SAM) is the methyl group donor for numerous reactions. The degradation product of SAM is S-adenosylhomocysteine (SAH), which is hydrolyzed to homocysteine by SAH-hydrolase. Homocysteine can be transsulfurated to cystathionine by the vitamin B6-dependent cystathionine beta-synthase (CBS), and subsequently, via the vitamin B6-dependent cystathionine gamma-lyase (CGL), to cysteine. Cysteine is essential for glutathione synthesis.(Finkelstein, 1990)

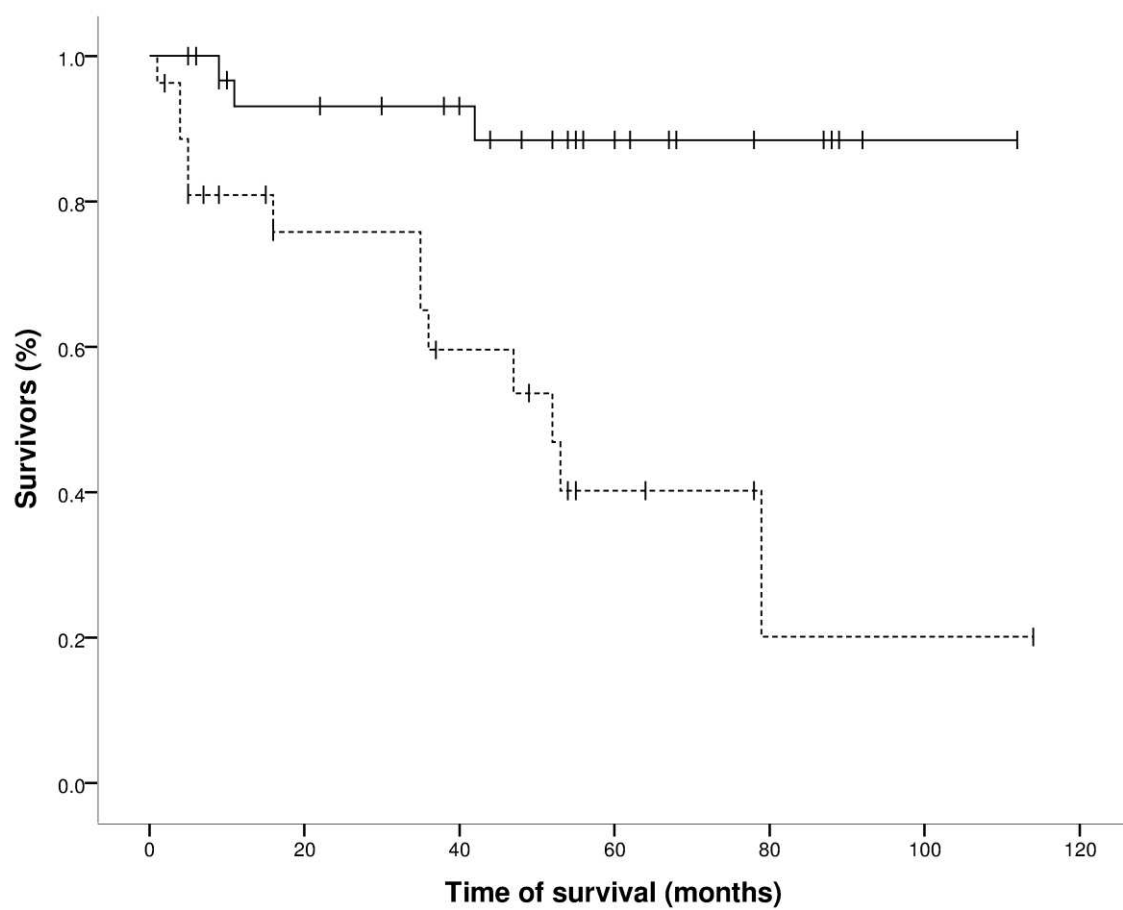


Figure 2a

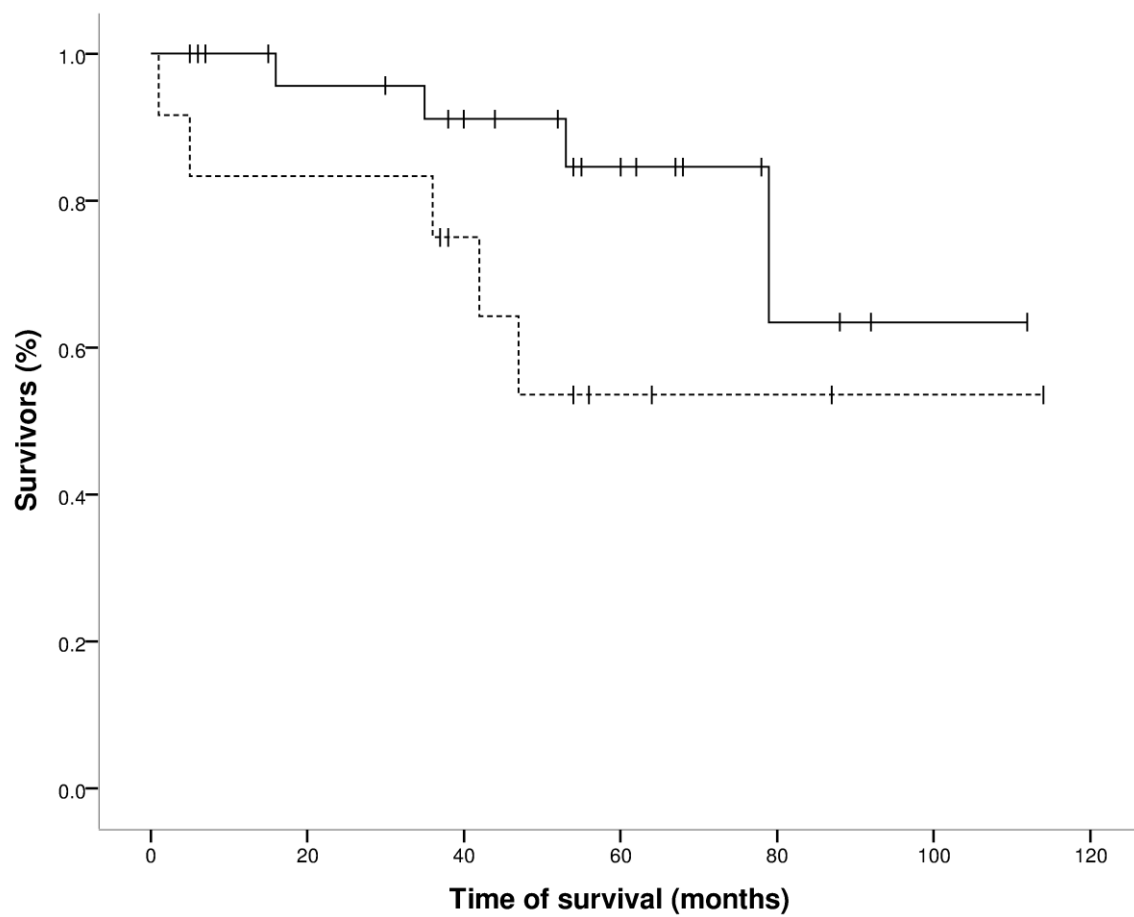


Figure 2b

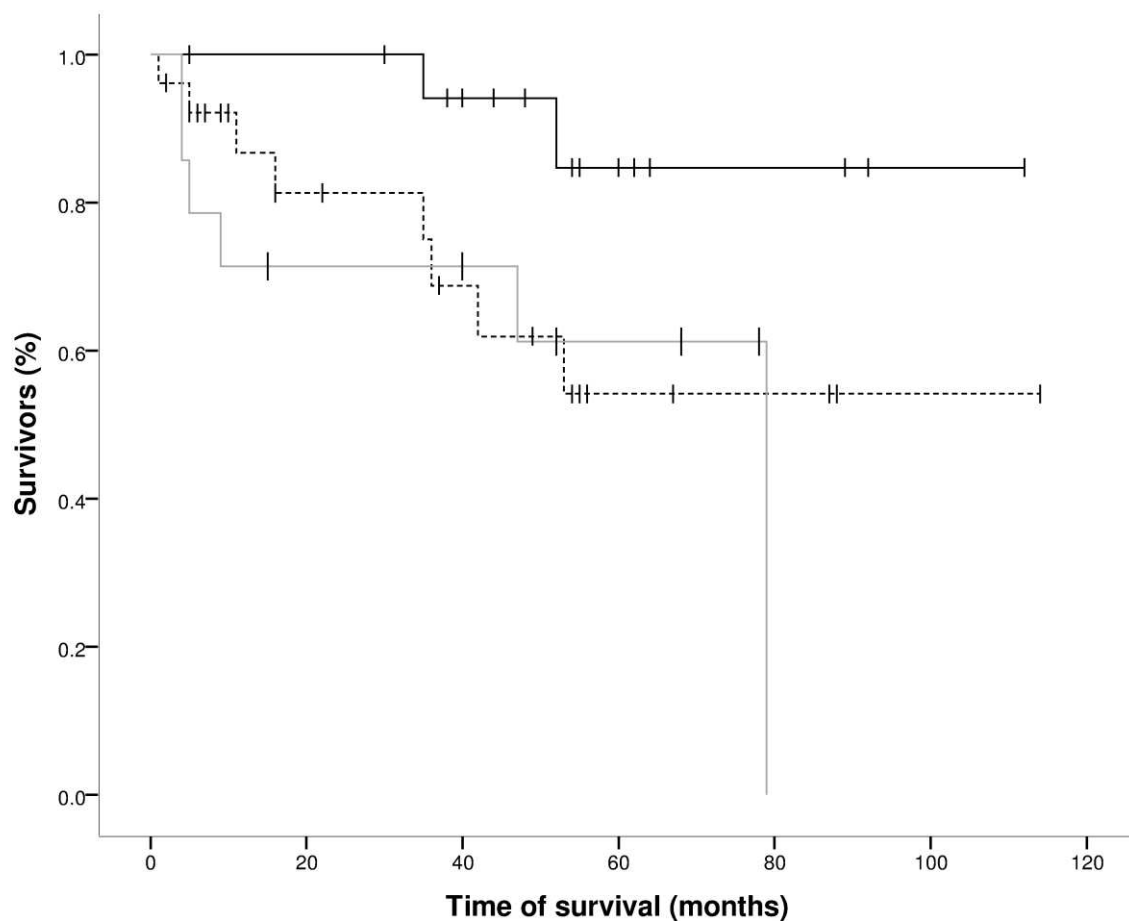


Figure 2c

Figure 2 – Age, Karnofsky performance score, Tc2 c.776C>G, and overall survival

Overall survival of patients grouped by a) age (<60 = black line, ≥60 years = broken line), b) Karnofsky performance score (< 70 = broken line, ≥ 70 = black line) and c) Tc2 c.776C>G genotype (CC = black line, CG = broken line, GG = grey line) is illustrated by Kaplan Meier curves. Censored cases are indicated by upright dashes. x-axis: months; y-axis: proportion of patients alive.

Tables

Table 1: Variants and mean overall survival (months)

Variant	Genotype and overall survival			COX-regression: Wald; p
CBS844ins68	del/del (.82)	ins/del (.18)	ins/ins (-)	
	81±7	56±9	not observed	1.1; 0.304
DHFR c.594+59del19bp	del/del (.20)	ins/del (.50)	ins/ins (.31)	
	87±13	78±10	60±9	2.0; 0.162
MTHFR c.677C>T	CC (.52)	CT (.36)	TT (.13)	
	76±10	64±8	101±12	1.0; 0.310
MTHFR c.1298A>C	AA (.53)	AC (.37)	CC (.10)	
	81±9	75±11	60±11	0.018; 0.892
MTR c.2756A>G	AA (.66)	AG/GG (.34)		
	79±8	79±11		0.38; 0.537
RFC1 c.80G>A	GG (.31)	AG (.42)	AA (.26)	
	78±12	72±11	68±5	0.69; 0.408
Tc2 c.776C>G	CC (.34)	CG (.44)	GG (.23)	
	100±8	76±11	52±10	7.3; 0.007

Median overall survival was not yet reached. Thus, mean overall survival in months \pm 1 standard deviation is depicted. P-values refer to multivariate analysis with age and gender and Karnofsky performance score before therapy as co-variables. The GG genotype of MTR c.2756A>G was

only observed in two patients. Thus, for statistical analysis, GG was pooled with AG.

Table 2: Transcobalamin c. 776C>G genotypes and mean Karnofsky scores

TC2	CC	CG	GG	ANOVA: F; p
Karnofsky score before treatment	77±12	71±18	70±17	0.62; 0.543
Karnofsky score after treatment	92±7	81±25	67±38	3.16; 0.054

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